

Streptococcus pneumoniae Proteins Released into Medium upon Inhibition of Cell Wall Biosynthesis

REGINE HAKENBECK,* CHRISTIANE MARTIN, AND GIOVANNA MORELLI

Max-Planck-Institut für Molekulare Genetik, D-1000 Berlin 33, Germany

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Inhibition of murein biosynthesis in *Streptococcus pneumoniae* by either penicillin or bacitracin leads to an increase in the amount of protein secreted into the medium. This process was studied in wild-type cells grown under lysis-permissive conditions as well as in an autolysin-deficient mutant. The time course of secretion did not follow cellular lysis but commenced immediately after the addition of the cell wall inhibitor in a manner similar to that described recently for cell wall and membrane components in various tolerant streptococci. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that this increase was not due to the stimulation of release of three protein components which are secreted under normal growth conditions; rather, a complex set of cellular proteins escaped from the antibiotic-treated pneumococci. The proteins released during bacitracin treatment was slightly different from those observed when penicillin was used. Analysis on sucrose gradients indicated that the secreted proteins were membrane bound rather than soluble. Membrane vesicles could indeed be detected by electron microscopy of negative-stained secreted material.

Recently, a novel effect of inhibition of peptidoglycan biosynthesis induced by various antibiotics in several species of gram-positive bacteria has been documented: immediately upon addition of the drug, large amounts of membrane components (lipids and lipoteichoic acid) and newly synthesized cell wall material (murein precursors and teichoic acid) are secreted into the medium (J. L. Brisette, G. D. Shockman, and R. A. Pieringer, Fed. Proc. **40**:1630, 1981; 12-15, 32, 34). This phenomenon is not due to lysis of the bacteria since autolysin-deficient and tolerant strains were used in these studies and the phenomenon could also be observed in wild-type cells grown under lysis-nonpermissive conditions.

The cause of release of these components remained unclear. Vesicular structures have been occasionally observed by electron microscopy near the surfaces of drug-treated cells, suggesting that when the expansion of the rigid cell wall layer is inhibited, the bacteria continue to synthesize membrane material and simply shed it into the medium (13).

To gain more information about this process, we analyzed the proteins that were secreted under the same conditions as described above by using an autolysin-deficient mutant of *Streptococcus pneumoniae*. The behavior of these proteins on sucrose gradients strongly supports the postulated formation of membrane vesicles, and their presence was revealed by electron

microscopy. Comparison with proteins derived from cytoplasm, membranes, and mesosomes by two-dimensional gel electrophoresis showed that the secreted proteins are most likely of mesosomal origin.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. pneumoniae* R6 is a derivative of the Rockefeller University laboratory strain. Strain cwI is an autolysin-deficient transformant of the wild type (17).

Bacteria were grown at 37°C without aeration in a chemically defined, enriched medium (A. Tomasz, Bacterial Proc., p. 28, 1964) with choline or ethanolamine. Yeast extract (0.1%, Difco Laboratories) was added to the growth medium of the mutant. When cells were labeled with [³⁵S]methionine or with [³H]phenylalanine plus [³H]lysine, the corresponding unlabeled amino acids were omitted from the medium in the case of the mutant (cwI) and were kept at a concentration of 4 µg/ml for the wild-type strain (R6). Growth was monitored by measuring the optical density at 560 nm.

Antibiotics. Penicillin G (Hoechst) was used at a final concentration of 0.07 µg/ml or 0.1 U/ml, and chloramphenicol (Serva) was used at a concentration of 25 µg/ml. Drugs were usually added at an optical density of 0.13. [³H]propionyl ampicillin was synthesized by the method of Schwarz et al. (28) and stored lyophilized at -60°C until use.

Biosynthetic labeling of pneumococcal proteins. Bacterial cultures were labeled with either L-[³⁵S]methionine (Amersham, 600 Ci/mmol; 50 to 60 µCi/ml of growth medium) or L-[ring-2,6-³H(N)]phenylalanine (New England Nuclear Corp., 60 Ci/mmol) plus L-[4,5-

$^3\text{H}(\text{N})$ lysine (75 Ci/mmol). The last two chemicals were both added at 200 $\mu\text{Ci/ml}$. Bacteria were labeled by one of two procedures. (i) Old label: The radioactive amino acid was added to a growing culture at about 5×10^6 cells per ml. After three generations, bacteria were centrifuged and washed once with isotope-free medium. They were suspended in prewarmed nonradioactive medium and allowed to grow for 10 min before the addition of antibiotics. (ii) New label: Antibiotics and radioactive compounds were added simultaneously. For preparation of labeled membranes and cytoplasmic or mesosomal proteins, cells were labeled for 1 h before harvesting.

Determination of radioactive cellular proteins and proteins secreted into medium. Samples were taken from a bacterial culture labeled with radioactive amino acids, either from the whole culture or from the growth medium after removal of the cells by centrifugation. Proteins were precipitated with 10% trichloroacetic acid, filtered onto Millipore filters (pore size, 0.2 μm), and dried. After the addition of 5 ml of toluene scintillator containing 0.25% 2,5-diphenyloxazole, the radioactivity retained on the filters was counted in a Searle Mark III liquid scintillation counter.

Preparation of proteins released into the growth medium. Bacteria from a growing culture were centrifuged ($28,000 \times g$, 15 min in a Sorvall centrifuge). The supernatant was carefully removed, dialyzed extensively against distilled water at 4°C , and lyophilized. The lyophilized protein sample was kept at -20°C until use.

Preparation of cytoplasmic and membrane proteins. Cells were harvested by centrifugation, washed once with potassium phosphate buffer (50 mM, pH 7.2), and suspended in the same buffer. They were broken by shaking with glass beads (diameter, 0.17 mm) for 20 min in a Mickle disintegrator. Unbroken cells and cell walls were removed by centrifugation at $35,000 \times g$ for 15 min in a Sorvall centrifuge, and membranes were separated from cytoplasmic proteins by centrifugation with a Ti 50.1 rotor (3 h, $160,000 \times g$) in a Beckman LS-50 ultracentrifuge. By this procedure, 98% of penicillin-binding proteins (PBPs), components of the cytoplasmic membrane, are found in the pellet (11). Membranes were washed twice with buffer and resuspended at a protein concentration of 30 to 50 mg/ml as determined by the method of Lowry et al. (21). Cytoplasm (supernatant fluid) and membranes were kept frozen at -20°C .

Preparation of mesosomes. Protoplasts were prepared by the procedure described by Calandra et al. (4), and mesosomes that were released during protoplasting (16) were collected by high-speed centrifugation of the protoplasting medium. [^{35}S]methionine-labeled cells of the mutant strain cwI (10 ml of culture) were pelleted at $35,000 \times g$ in a Sorvall centrifuge, washed once in Tris-maleate buffer (50 mM, pH 6.8), and suspended in 150 μl of protoplasting medium (25% raffinose, 20 mM MgSO_4 , 50 mM Tris-maleate, pH 6.8). Crude autolysin (15 μl) from wild-type pneumococci (33) was added, and the cell suspension was incubated at 37°C . Protoplasting was complete after 80 min as judged by light microscopy. Protoplasts were centrifuged in an Eppendorf centrifuge for 10 min, and the supernatant fluid was carefully removed and centrifuged again under the same conditions. The supernatant fluid was collected, 2 ml of Tris-maleate buffer

was added, and the supernatant was centrifuged in a Ti 50.1 rotor at $160,000 \times g$ for 3 h. The pellet (mesosomal membranes) was suspended in 100 μl of Tris-maleate buffer and stored at -20°C until use.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli and Favre (18). The concentrations were 5% acrylamide and 0.13% *N,N'*-methylenebisacrylamide for the stacking gel and 10% acrylamide and 0.13% *N,N'*-methylenebisacrylamide for the separating gel. Protein samples containing 1×10^5 to 5×10^5 cpm or up to 100 μg of protein were loaded. Gels were prepared for fluorography as described by Bonner and Laskey (2) with En^3Hance (New England Nuclear), and fluorograms were produced with Kodak X-Omat film after presensitization of the film (19).

Two-dimensional gel electrophoresis. The method of isoelectric focusing on slab gels described by Ferro-Luzzi Ames and Nikaido (6) was modified; to obtain a linear pH gradient from pH 4 to 7, 2% ampholines (LKB) were used with a pH 3.5 to 10:pH 4 to 6:pH 5 to 7 ratio of 2:3:4. For the second dimension, we used SDS-PAGE with a 10% separating gel as described above.

Identification of PBPs. PBPs in membranes (100 μg of proteins in 5 μl) were labeled with [^3H]propionyl ampicillin (5×10^5 cpm) during 20 min of incubation at 37°C . Samples were prepared for SDS-PAGE, and PBPs were visualized after fluorography of the gels (11).

Sucrose gradient centrifugation. Radioactive protein samples were layered on top of a linear sucrose gradient (5 to 40%) with a 0.5-ml cushion of 55% sucrose at the bottom of the nitrocellulose tube. Gradients were centrifuged for 14 h at 38,000 rpm in an SW41 rotor. Fractions were collected after puncturing the bottom of the tube, and radioactivity was determined after trichloroacetic acid precipitation as described above.

Electron microscopy. Cells (10 ml of culture) were collected on a Millipore filter (pore size, 0.45 μm), fixed in a 2% glutaraldehyde, and postfixed with 1% osmium tetroxide at 4°C . Staining with 5% uranyl acetate for 1 h was followed by ethanol dehydration. Infiltration and embedding with Epon were carried out by standard procedures. Thin sections were obtained with an OMU3 Microtom (Reichert, Austria), post-stained with lead citrate (25), and viewed under a Philips EM 301 electron microscope. The secreted material, which was pelleted from the medium of penicillin-treated bacteria (10 ml of culture, 1 h of penicillin treatment) in a Ti 50.1 rotor ($160,000 \times g$, 3 h), was observed after negative staining with 2% phosphotungstic acid.

RESULTS

Release of [^{35}S]methionine-labeled macromolecular material during inhibition of cell wall biosynthesis. Radioactive methionine and the antibiotic were added at the same time. Soon after the drug was added, a small but distinct increase in the amount of labeled macromolecular material in the medium was apparent (Fig. 1).

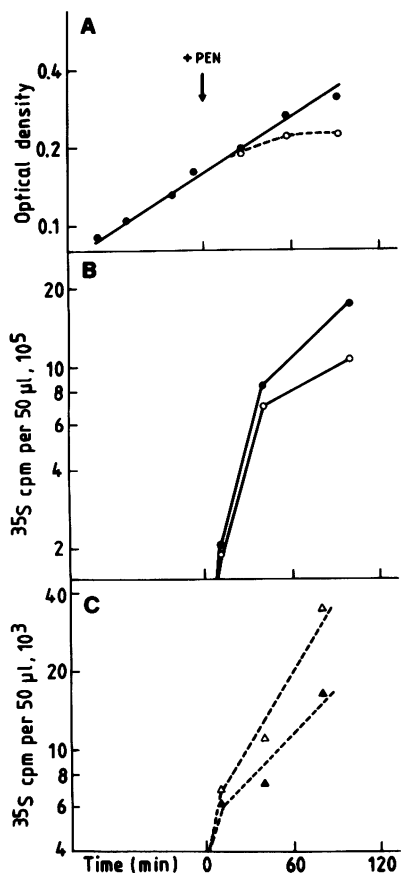


FIG. 1. Growth, incorporation of [35 S]methionine, and release of labeled macromolecular material into the growth medium of penicillin treated and untreated autolysin-defective mutant *cw1*. Growth was followed by measuring optical density (A). Total incorporation was determined by cold trichloroacetic acid precipitation with 50- μ l samples of the cultures (B) and of the medium (C). Penicillin (0.07 μ g/ml) and [35 S]methionine were added simultaneously as depicted by the arrow (+ PEN; ○, △). A control culture received no penicillin (●, ▲).

As shown in Table 1, this process was observed in the wild-type strain grown under lysis-nonpermissive conditions as well as in the autolysin-deficient mutant, and it was seen both when the cells were labeled before the antibiotic treatment and when they were labeled during it. The release was not restricted to penicillin, which inhibits cell wall biosynthesis at a late stage, but also occurred with early inhibitors like bacitracin.

Analysis of secreted labeled proteins from penicillin-treated and untreated cultures. A comparison by SDS-PAGE between [35 S]methionine-labeled proteins obtained from cultures of penicillin-treated and untreated pneumococci

showed few proteins in the medium without the addition of antibiotic (Fig. 2, lanes 1 and 4). Three components were present almost exclusively when the new labeling method was applied. They appear to represent secretory proteins of pneumococci (I, II, and III). In the presence of penicillin, however, these proteins were not released in higher amounts, but a different and complex set of proteins was released (Fig. 2, lanes 2 and 3). The three proteins mentioned above were also present only in the new-labeled material. All other penicillin-induced secreted proteins were similar in terms of quality, whether they were derived from old- or new-labeled cultures. Raising the concentration of penicillin up to 100 times the minimal inhibitory concentration did not change the protein pattern (data not shown). If proteins were pulse-labeled at different times during penicillin treatment, clearly all components in the medium were labeled at 10 min and to a lesser extent at 80 min after the addition of the antibiotic (Fig. 3, lanes 1 and 2).

Effect of chloramphenicol on penicillin-induced secretion. In amino acid-starved bacteria, penicillin can no longer act as a lytic and bactericidal agent (5, 20). Chloramphenicol reduces but does not prevent penicillin-induced secretion of cell surface components (34; R. Hakenbeck, D. Horne, and A. Tomasz, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 18th, Atlanta, Ga., abstr. no. 250, 1978). We have investigated the release of proteins in old-

TABLE 1. Release of [35 S]methionine-labeled proteins during inhibition of cell wall biosynthesis^a

Antibiotic ^b	Protein released (% of total) by strain:			
	cw1		R6 ^c	
	Old	New	Old	New
None	0.9	1.3	1.5	1.3
Penicillin				
10 \times MIC	1.8	2.4	2.5	2.0
100 \times MIC	2.0			
Penicillin plus chloramphenicol	1.45			
Bacitracin	1.4	2.6	2.2	2.7

^a Cells were grown and labeled with [35 S]methionine by the procedures for biosynthetically old or new proteins (see text). Proteins released into the medium were determined 40 min after drug addition.

^b Concentrations used: penicillin, 10 \times MIC (minimal inhibitory concentration), 0.07 μ g/ml; bacitracin, 10 μ g/ml; chloramphenicol, 25 μ g/ml.

^c Wild-type strain R6, grown under lysis-nonpermissive conditions in ethanolamine-containing medium.

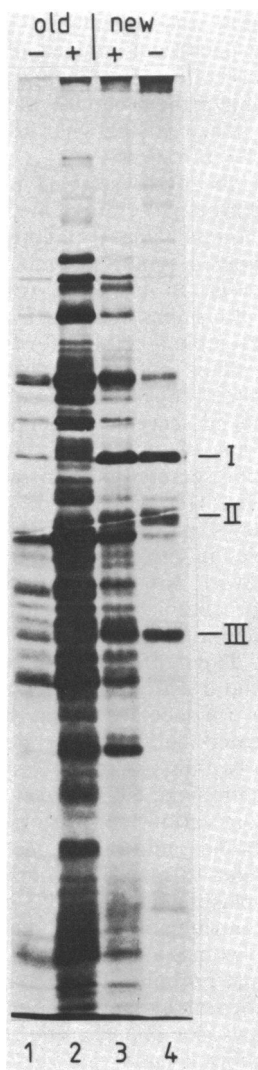


FIG. 2. SDS-PAGE of [35 S]methionine-labeled secreted proteins derived from penicillin-treated and untreated *S. pneumoniae* cwI. Exponentially growing cultures were labeled before (old) or during (new) penicillin treatment. Control cultures received no antibiotic (-). At 60 min after drug addition, cells were removed by centrifugation, and the supernatants were dialyzed and lyophilized as described in the text. Samples representing 1 ml of culture medium (old) or 0.5 ml of medium (new) were loaded on a 10% gel for SDS-PAGE. Proteins were visualized on X-ray film after fluorography of the gel.

labeled pneumococci under conditions in which chloramphenicol and penicillin were added simultaneously. Table 1 shows the quantitative aspect of secretion of the experiment: the addition of cloramphenicol led to a limited escape of proteins. The secreted proteins were analyzed

by SDS-PAGE (Fig. 3). Old-labeled proteins derived from cells treated with chloramphenicol plus penicillin were compared with the patterns of new-labeled secreted proteins. The composition of the molecules was the same in both lanes (except for the secretory proteins I through III in the new-labeled sample), but the amount in the presence of chloramphenicol was clearly diminished.

Comparison between penicillin- and bacitracin-induced secreted proteins. We examined the new labeled secreted proteins from bacitracin-treated

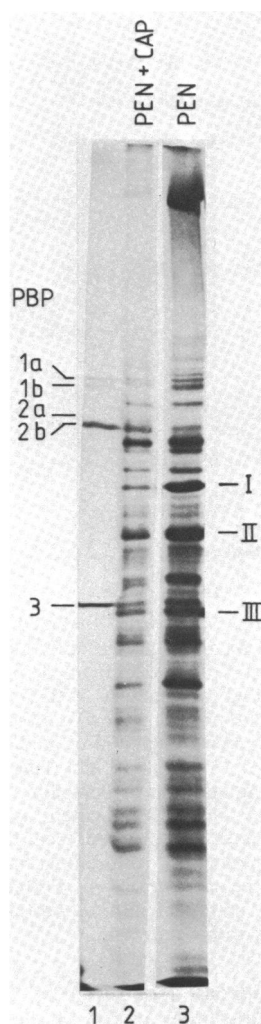


FIG. 3. Effect of chloramphenicol on penicillin-induced secretion. Exponentially growing cells of *S. pneumoniae* cwI were labeled with [35 S]methionine and treated with penicillin (PEN) and chloramphenicol (CAP) as shown. Supernatant fluids were collected 60 min after drug treatment. Each sample represents protein of 1 ml of culture medium. Lane 1 represents PBPs of *S. pneumoniae*, labeled with [3 H]propionyl-ampicillin in 100 μ g of membrane protein.

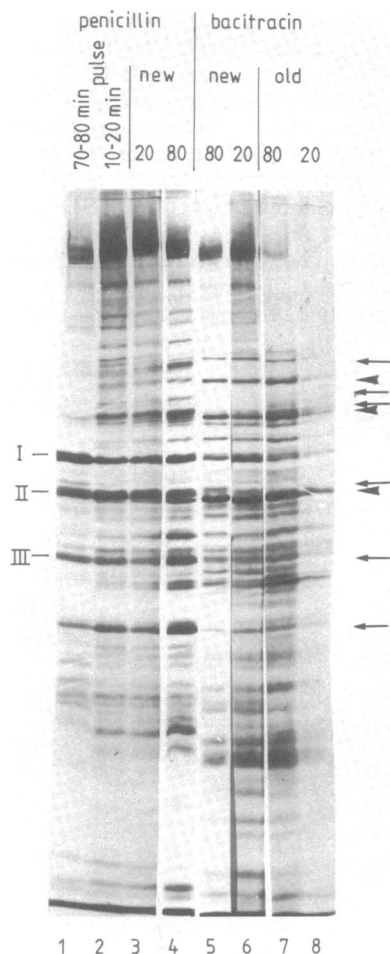


FIG. 4. Comparison of [^{35}S]labeled proteins released into the medium during inhibition of cell wall biosynthesis with either bacitracin or penicillin. The mutant strain *cwl* was labeled with [^{35}S]methionine either before (old) or during (new) antibiotic treatment (bacitracin, 10 $\mu\text{g}/\text{ml}$). Penicillin-treated cultures were pulse-labeled for 10 min at 10 min (lane 2) or 70 min (lane 1) after the addition of penicillin. Supernatants were isolated at the times indicated, as described in the legend to Fig. 2. Samples represent the equivalent of 1 ml of supernatant. Autoradiograms have been arranged from the same gel. \leftarrow , Proteins present in higher amounts with penicillin; \blacktriangleleft , proteins present in higher amounts with bacitracin.

and penicillin-treated cultures by SDS-PAGE. The four middle lanes in Fig. 4 represent samples taken at 20 and 80 min after the addition of either drug. They reveal surprising differences between the two conditions. Some protein components were present in much higher amounts or even exclusively when penicillin was used. For others, particularly high-molecular-weight proteins, the opposite was true. These differences were present regardless of the time at which the

culture medium was collected. Fig. 4, lanes 7 and 8, show bacitracin-induced old-labeled secreted proteins collected at 20 or 80 min. At both times, the same protein pattern was found, and no major difference existed when compared with the new-labeled proteins.

Cellular origin of the secreted proteins. The early onset of protein secretion after the addition of the antibiotics made it unlikely that this reflected simply a minor portion of the cells undergoing lysis. If this were true, then the composition of proteins obtained from the medium should resemble a cell lysate. In comparing membrane proteins, cytoplasmic proteins, and a whole cell lysate prepared from control cells to penicillin-induced secreted proteins, no overall similarity to any of the cell fractions could be detected for the secreted proteins after analysis by SDS-PAGE. This was true of samples prepared from new- and old-labeled cultures and of proteins labeled in vitro with [^{14}C]formaldehyde (data not shown). No major difference among membrane (or cytoplasmic) preparations from control, penicillin-treated, and bacitracin-treated cells was detected; the only exception observed was the drastic reduction of one membrane protein (molecular weight, 60,000) from bacitracin-treated cultures. We performed an analysis on a two-dimensional gel system, comparing [^{35}S]methionine-labeled released proteins with those from various cellular fractions: cytoplasm, membranes, and mesosomes. By mesosomes, we refer to material which is released during protoplasting and can be collected by high-speed centrifugation from the medium in which protoplasting occurs (16, 27). Membrane and mesosomal proteins (Fig. 5B and C) show clear similarities each other, although few major spots in the mesosomal preparation were absent from the membranes and vice versa. No correspondence of these two fractions to cytoplasmic proteins (Fig. 5A) can be detected. A strong correlation exists between mesosomal and released proteins (Fig. 5D). Some radioactive spots which are present in membranes, mesosomes, and secreted proteins are indicated in Fig. 5B through D to facilitate orientation on the two-dimensional gels. The three secretory proteins, which are marked by arrows in Fig. 5D, were absent from the cellular fractions.

Figure 3 suggests the presence of other, minor membrane components among the released proteins. When compared with the PBPs in pneumococci, radioactive bands which comigrate at the same molecular size with the PBPs are visible. In fact, when unlabeled secreted material from bacitracin-treated cultures was labeled with [^3H]propionyl ampicillin, PBPs could be detected after fluorography on SDS gels and in membranes isolated from these cultures about

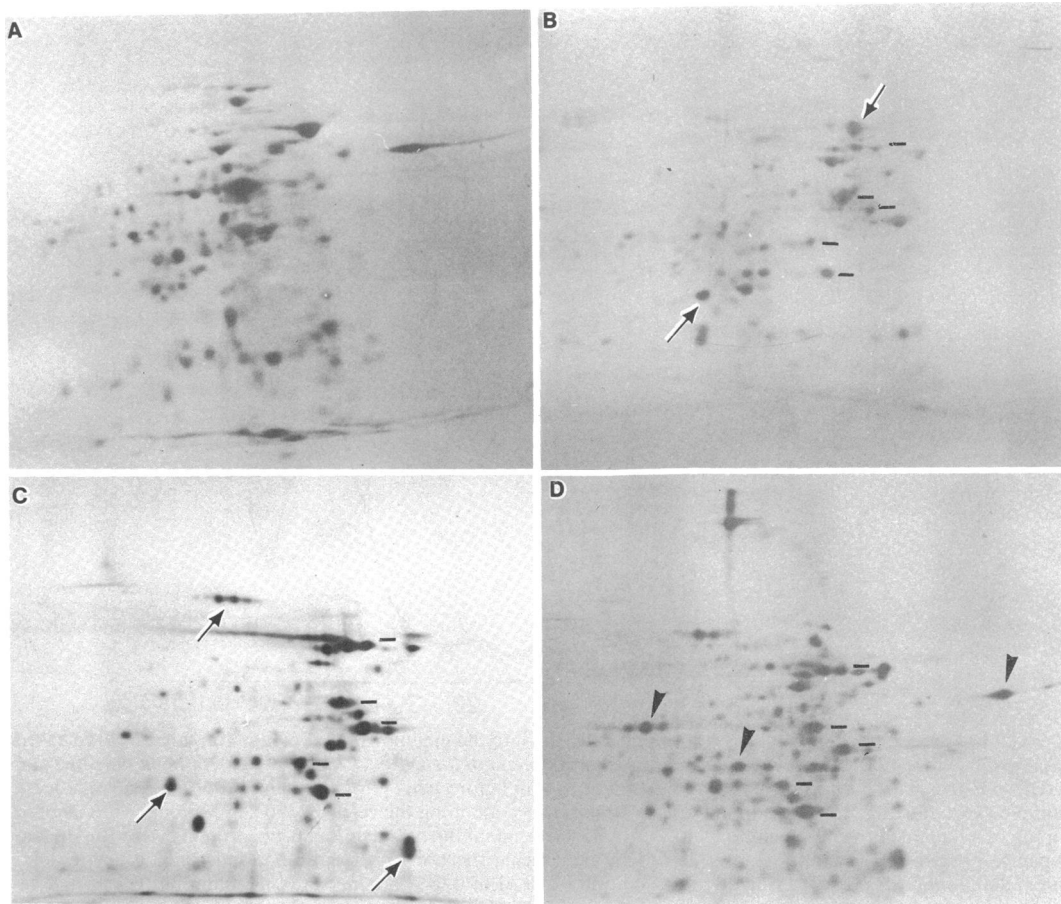


FIG. 5. Comparison of secreted proteins to membrane, cytoplasmic, and mesosomal proteins by two-dimensional gel electrophoresis. (A) Cytoplasmic proteins; (B) membrane proteins; (C) mesosomal proteins; (D) secreted proteins. The secretory proteins I, II, and III are marked by arrowheads in D. Some proteins present in B, C, and D are indicated by lines. Proteins specifically present either in the mesosomal preparation or in the membranes but not in both fractions are indicated by arrows in B and C. Proteins released into the medium were collected after 60 min of penicillin treatment and labeling with [35 S]methionine (new label). [35 S]methionine-labeled membranes, cytoplasmic fraction, and mesosomes were prepared from untreated cells. Samples containing about 5×10^5 cpm were separated in the first dimension by isoelectric focusing and in the second dimension by SDS-PAGE. Radioactive proteins were visualized after fluorography.

30% less PBPs were detectable if compared with membranes from untreated cells (data not shown). A similar phenomenon, namely the release of PBPs and a simultaneous decrease of these proteins in the cells, has been observed in group A streptococci during penicillin treatment (10).

Native size of secreted proteins. From an old-labeled penicillin-treated culture, the cells were removed 60 min after the addition of the antibiotic, and part of the supernatant was loaded directly on a linear sucrose gradient. The sedimentation behavior was analyzed in comparison with membrane and cytoplasmic proteins (Fig. 6). The secreted material banded at the same position as membrane proteins, shifting towards

smaller particles, a behavior which has been shown for mesosomes also (16). The cytoplasmic proteins remained on top of the gradient. This agrees with the conception that the antibiotic-induced secreted proteins are assembled in membrane vesicles.

Electron microscopy of the secreted material. To verify our results obtained above, we tried to identify the presumptive membrane vesicles by electron microscopy during the secretion process after thin sectioning of penicillin-treated bacteria and by negative staining of sedimentable secreted material (Fig. 7). In Fig. 7B and C, vesicular structures extruding from the cell surface of antibiotic-treated pneumococci can be seen, and these structures were completely ab-

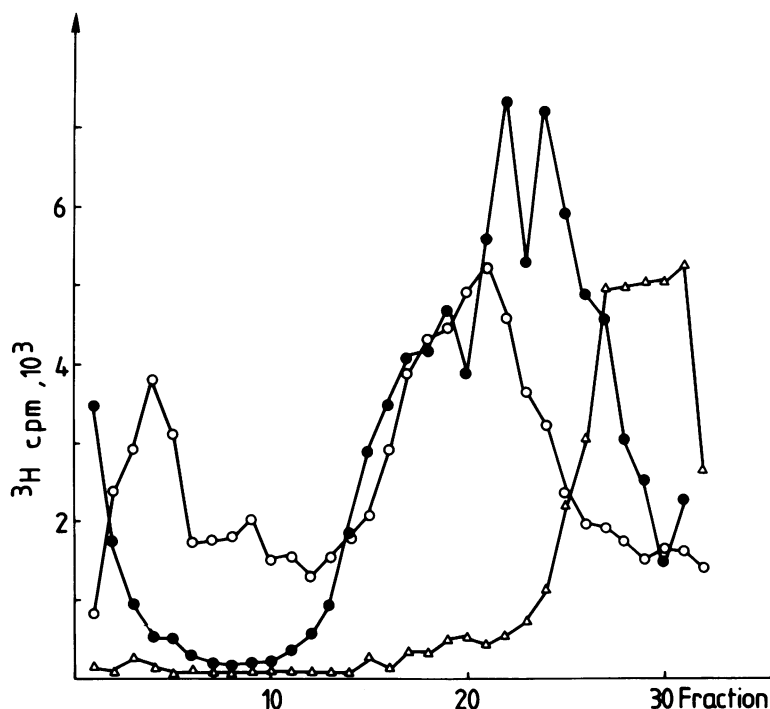


FIG. 6. Sucrose gradient analysis of proteins released into the medium after penicillin treatment. (●) released proteins; (○) membranes; (△) cytoplasmic proteins. *S. pneumoniae* cwI was labeled with [^3H]phenylalanine and [^3H]lysine and chased into nonradioactive medium 10 min before penicillin treatment. After 60 min, cells were removed by centrifugation, and 0.6 ml of the supernatant containing the released labeled proteins were layered on top of a linear sucrose gradient (5 to 40%). Radioactive membranes and cytoplasmic proteins were run on two separate gradients. Fractions were collected by puncturing the bottom of the tube, and trichloroacetic acid-precipitable material was determined after addition of 10 μl of 0.04% bovine serum albumin per fraction.

sent from untreated control cells (Fig. 7A). After removal of the penicillin-treated cells, these vesicles could be pelleted by high-speed centrifugation of the medium. Negative stain of these structures is shown in Fig. 7D.

DISCUSSION

The results presented in this study show that inhibition of cell wall biosynthesis leads to the release of a specific subset of proteins. The properties of protein release follow the observations made by Tomasz and coworkers for cell wall components: an early onset of release after the addition of antibiotics (Fig. 1), reduction of release by additional chloramphenicol treatment (Fig. 4), and its manifestation during treatment with different inhibitors of murein biosynthesis (Table 1; 12, 13, 34). These proteins are aggregated in membrane vesicles and closely resemble mesosomal proteins. This confirms the hypothesis which was formulated in previous reports of Tomasz and his group, namely, that antibiotics which directly prevent the synthesis of murein cause shedding of membrane vesicles

analogous to the process of pinocytosis in eucaryotic cells (13). The secretions of lipids, lipoteichoic acid, and proteins are, therefore, different manifestations of one and the same phenomenon.

One puzzling observation made in the present study is the release during bacitracin treatment of certain proteins that differed in quantity from those derived from penicillin-treated cultures. The presumption has been made that the formation of complexes between this antibiotic and undecaprenyl phosphate can result in a disruption of protoplast membranes (29). It is possible that such a complex might also lead to structural changes in the membranes of the secreted vesicles.

Another, more important, aspect concerns the origin of the secreted proteins. When analyzed on a two-dimensional gel system in comparison with cytoplasmic, membrane, and mesosomal proteins, most of the major labeled secreted components resemble membrane proteins, but they resemble mesosomal proteins even more closely. The interpretation that the vesicular structures which are released during inhibition

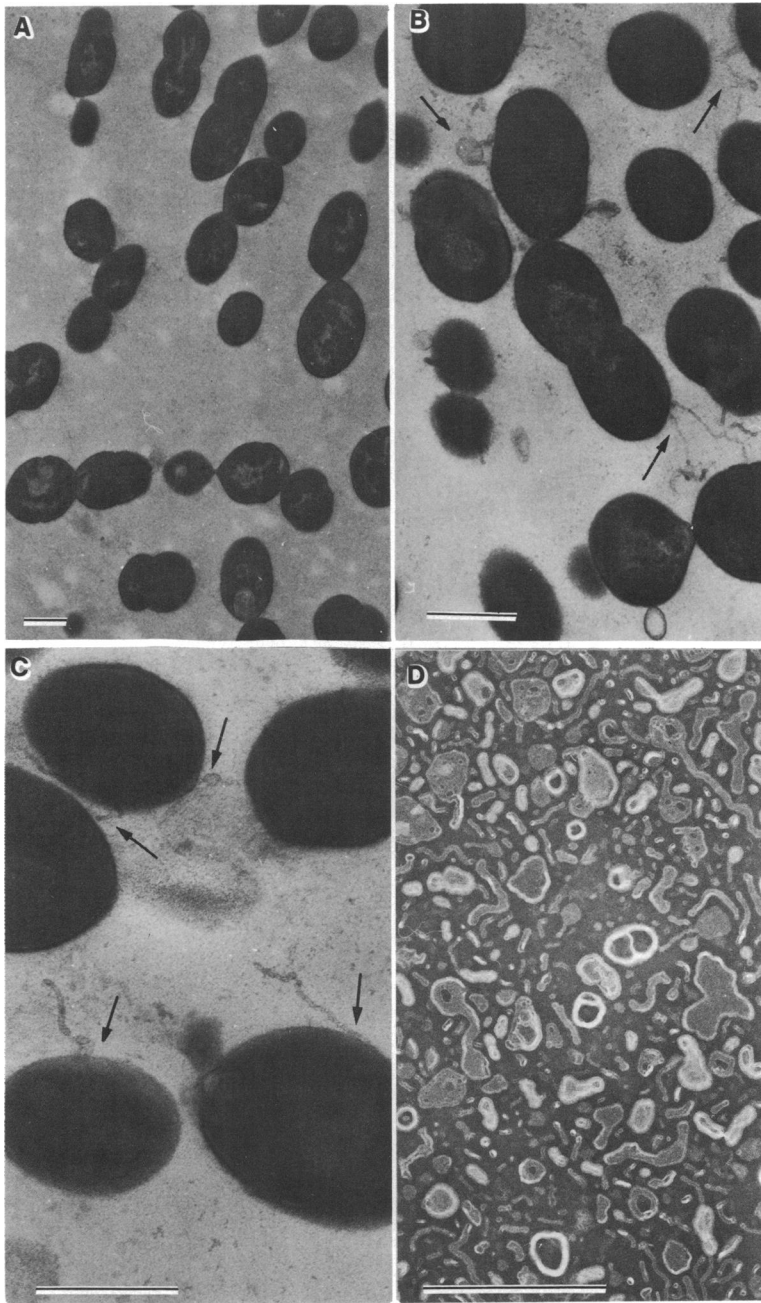


FIG. 7. Electron microscopy of vesicles released from penicillin-treated *S. pneumoniae* cwI. (A) Thin section of untreated control cells; (B, C) thin sections of penicillin-treated cells (60 min, 0.07 µg/ml) with vesicles extruding from the surface (arrows); (D) negative stain of vesicles isolated by high-speed centrifugation from growth medium of penicillin-treated cells. Bars, 0.5 µm.

of murein biosynthesis are in fact mesosomes protruding from the drug-treated bacteria would explain the specific make-up of the vesicles. Considerable alterations in protein composition of mesosomal membranes compared with the

cytoplasmic membrane have been detected in several gram-positive species (22, 24, 30). These structures are easily released, e.g., during protoplast formation induced by lytic enzymes (3, 7-9, 16, 23, 27). Mesosomes are located near the

septum of the cell (for review, see reference 26) and are well known in *S. pneumoniae* (31). In pneumococci, cell wall synthesis takes place at the equatorial plane of the bacteria (1), and interference with its biosynthesis could easily have a primary effect on the mesosomal membranes being proximal to the division zone. Since no specific biochemical markers for mesosomes in pneumococci are known at present, the proof of this hypothesis has to await further experiments.

The bacteria seem to continue the synthesis of this particular membrane material at the time when murein biosynthesis is greatly inhibited. Why and how this material is directed outside the cell is still unclear. It should be pointed out that no autolytic activity is involved in this process, but it occurs under conditions in which the cells contain either a mutated autolysin (strain cw1) or an inactive autolysin (wild-type strain grown in ethanolamine-containing medium) and therefore show tolerance against the lytic activity of inhibitors of cell wall biosynthesis. The release of membranous vesicles is merely the result of the biosynthetic halt imposed on the cells. This is different from the formation of protoplasts, which results in the release of mesosomes and in which cells are forced to lose the integrity of the murein layer by the action of lytic enzymes.

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LITERATURE CITED

- Barak, E. A., and A. Tomasz. 1970. Radioautographic evidence for equatorial wall growth in a gram-positive bacterium: segregation of choline-³H-labelled teichoic acid. *J. Cell Biol.* 47:786-790.
- Bonner, W. M., and R. A. Laskey. 1974. Fluorographic detection of ³H, ¹⁴C, and ³⁵S in acrylamide gels. *Eur. J. Biochem.* 46:83-88.
- Burdett, I. D. J., and H. J. Rogers. 1970. Modification of the appearance of mesosomes in sections of *Bacillus licheniformis* according to the fixation procedure. *J. Ultrastruct. Res.* 30:354-367.
- Calandra, G. B., K. M. Nugent, and R. M. Cole. 1975. Preparation of protoplasts of group H streptococci (*Streptococcus sanguis*). *Appl. Microbiol.* 29:90-93.
- Davis, B. D. 1950. Studies on nutritionally deficient bacterial mutants isolated by means of penicillin. *Experientia* 6:41-80.
- Ferro-Luzi Ames, G., and K. Nikaido. 1976. Two-dimensional gel electrophoresis of membrane proteins. *Biochemistry* 15:616-623.
- Fitz-James, P. C. 1968. The collection of mesosome vesicles extruded during protoplasting, p. 124-143. In L. B. Guze (ed.), *Microbial protoplasts, spheroplasts, and L-forms*. The Williams & Wilkins Co., Baltimore.
- Ghosh, B. K., and R. G. E. Murray. 1969. Fractionation and characterization of the plasma and mesosome membrane of *Listeria monocytogenes*. *J. Bacteriol.* 97:426-440.
- Greenawalt, J. W., and T. L. Whiteside. 1975. Mesosomes: membranous bacterial organelles. *Bacteriol. Rev.* 39:405-463.
- Gutman, L., R. Williamson, and A. Tomasz. 1981. Physiological properties of penicillin-binding proteins in group A streptococci. *Antimicrob. Agents Chemother.* 19:872-880.
- Hakenbeck, R., M. Tarpay, and A. Tomasz. 1980. Multiple changes of penicillin-binding proteins in penicillin-resistant clinical isolates of *Streptococcus pneumoniae*. *Antimicrob. Agents Chemother.* 17:364-371.
- Hakenbeck, R., S. Waks, and A. Tomasz. 1978. Characterization of cell wall polymers secreted into the growth medium of lysis-defective pneumococci during treatment with penicillin and other inhibitors of cell wall synthesis. *Antimicrob. Agents Chemother.* 13:302-311.
- Horne, D., R. Hakenbeck, and A. Tomasz. 1977. Secretion of lipids induced by inhibition of peptidoglycan synthesis in Streptococci. *J. Bacteriol.* 132:704-717.
- Horne, D., and A. Tomasz. 1977. Tolerant response of *Streptococcus sanguis* to beta-lactams and other cell wall inhibitors. *Antimicrob. Agents Chemother.* 11:888-896.
- Horne, D., and A. Tomasz. 1979. Release of lipoteichoic acid from *Streptococcus sanguis*: stimulation of release during penicillin treatment. *J. Bacteriol.* 137:1180-1184.
- Kusaka, I. 1975. Degradation of phospholipid and release of diglyceride-rich membrane vesicles during protoplast formation in certain gram-positive bacteria. *J. Bacteriol.* 121:1173-1179.
- Lacks, S. 1970. Mutants of *Diplococcus pneumoniae* that lack deoxyribonucleases and other activities possibly pertinent to genetic transformation. *J. Bacteriol.* 101:373-381.
- Laemmli, U. K., and M. Favre. 1973. Maturation of the head of bacteriophage T4. I. DNA packaging events. *J. Mol. Biol.* 80:575-599.
- Laskey, R. A., and A. D. Mills. 1975. Quantitative film detection of ³H and ¹⁴C in polyacrylamide gels by fluorography. *Eur. J. Biochem.* 56:335-341.
- Lederberg, J., and N. Zinder. 1948. Concentration of biochemical mutants of bacteria with penicillin. *J. Am. Chem. Soc.* 70:4267-4268.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- Owen, P., and J. H. Freer. 1972. Isolation and properties of mesosomal membrane fractions from *Micrococcus lysodeikticus*. *Biochem. J.* 129:907-917.
- Popkin, T. J., T. S. Theodore, and R. M. Cole. 1971. Electron microscopy during release and purification of mesosomal vesicles and protoplast membranes from *Staphylococcus aureus*. *J. Bacteriol.* 107:907-917.
- Reaveley, D. A. 1968. The isolation and characterization of cytoplasmic membranes and mesosomes of *Bacillus licheniformis* 6346. *Biochem. Biophys. Res. Commun.* 30:649-655.
- Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* 17:208-212.
- Rogers, H. J., H. R. Perkins, and J. B. Ward. 1980. Microbial cell walls and membranes. Chapman and Hall, London.
- Ryter, A., C. Fréhel, and B. Ferrandes. 1967. Compartiment des mesosomes lors de l'attaque de *Bacillus subtilis* par le lysozyme en milieu hyper ou hypotonique. *C. R. Acad. Sci.* 265:1259-1262.
- Schwarz, U., K. Seeger, F. Wengenmayr, and H. Strecker. 1981. Penicillin-binding proteins of *Escherichia coli* identified with a ¹²⁵I-derivative of ampicillin. *FEMS Microbiol. Lett.* 10:107-109.
- Storm, D. R., and J. L. Strominger. 1974. Binding of bacitracin to cells and protoplasts of *Micrococcus lysodeikticus*. *J. Biol. Chem.* 249:1823-1827.
- Theodore, T. S., and C. Panos. 1973. Protein and fatty

- acid composition of mesosomal vesicles and plasma membranes of *Staphylococcus aureus*. *J. Bacteriol.* **116**:571–576.
31. Tomasz, A., J. D. Janieson, and F. Ottolenghi. 1964. The fine structure of *Diplococcus pneumoniae*. *J. Cell Biol.* **22**:453–467.
32. Tomasz, A., and S. Waks. 1975. Mechanism of action of penicillin: triggering of the pneumococcal autolytic enzyme by inhibitors of cell wall synthesis. *Proc. Natl. Acad. Sci. U.S.A.* **72**:4162–4166.
33. Tomasz, A., and M. Westphal. 1971. Abnormal autolytic enzyme in a pneumococcus with altered teichoic acid composition. *Proc. Natl. Acad. Sci. U.S.A.* **68**:2627–2630.
34. Waks, S., and A. Tomasz. 1978. Secretion of cell wall polymers into the growth medium of lysis-defective pneumococci during treatment with penicillin and other inhibitors of cell wall synthesis. *Antimicrob. Agents Chemother.* **13**:293–301.